

A genetic method to differentiate *Petromyzon marinus* ammocoetes from those of the paired species *Lampetra fluviatilis* and *L. planeri*

by

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Genetic test

Abstract. – *Petromyzon marinus* and *Lampetra* sp. are protected by international conventions (Bern, 1979; Habitats directive, 1992). To estimate the abundance, and thus the precariousness of each population, it is advisable to distinguish the larvae of each species, because they may use the same environments in rivers. However, these larvae were indistinguishable on morphological criteria (oral disk, pigmentation, myomeres,...). We used a molecular method for discriminating between *P. marinus* and *Lampetra* sp. A 560 bp DNA fragment of the mitochondrial cytochrome b (*cyt b*) gene was amplified by PCR. Restriction fragment length polymorphism (RFLP) analysis was performed using selected restriction enzymes. This test is useful for discriminating young lamprey larvae less 60 mm total length, which are frequently mixed in ammocoete habitats. Moreover, this developed test can be realized with 3 mm² tissues (small piece of fin) only and does not require larvae sacrifice.

Résumé. – Une méthode génétique pour discriminer les ammocoetes de *Petromyzon marinus* et celles du complexe *Lampetra fluviatilis*-*L. planeri*.

Petromyzon marinus et *Lampetra* sp. sont actuellement des espèces protégées par les conventions internationales. Pour évaluer l'abondance des populations de ces espèces, il convient de distinguer les larves de chaque espèce car elles sont susceptibles d'utiliser les mêmes habitats. Or, ces larves ne peuvent être distinguées par des critères anatomo-morphologiques classiques (disque buccal, pigmentation, myomères,...). Une méthode moléculaire de discrimination entre *Petromyzon marinus* et *Lampetra* sp. a été mise au point. Un fragment d'ADN du gène codant le cytochrome b (*cyt b*) mitochondrial a été amplifié par PCR. Un fragment de 560 pb a été obtenu, puis digéré à l'aide d'enzymes de restriction. Ce test s'est avéré utile pour identifier avec certitude les larves des plus jeunes lamproies (taille inférieure à 60 mm) fréquemment mélangées dans les lits à ammocètes. En outre, cette méthode peut être mise en œuvre avec seulement 3 mm² de tissus (petit morceau de nageoire) et ne nécessite donc pas le sacrifice des larves testées. Son application dans le cadre de la gestion des populations de lamproies pourrait ainsi être envisagée.

In the northern hemisphere, lampreys are a single family, Petromyzontidae, with nine genera (Nelson, 1994). Three species *Petromyzon marinus* Linnaeus, 1758 (sea lamprey), *Lampetra fluviatilis* (Linnaeus, 1758) (river lamprey) and *Lampetra planeri* (Bloch, 1784) (brook lamprey) live in European rivers. *P. marinus*, *L. fluviatilis* and *L. planeri* are protected species (Bern Convention 09-19-1979) and also species of patrimonial and economical interests, whose conservation requires the designation of special conservation areas (Annex II of the EC Habitats Directive-92/43/CEE). Larvae (= ammocoetes) of different species may sometimes be found in the same rivers or even the same sites (Hardisty, 1969; Hardisty, 1970; Hardisty and Huggins, 1970), where interspecific competition appears to act similarly to intraspecific competition (Murdoch *et al.*, 1991). Lasne *et al.* (2010) report a high frequency (54%) of communal spawning involving both species in the *Lampetra* redds of a French coastal river (Oir).

As adults, identification of lamprey is chiefly based on the arrangement and character of the teeth on the mouth disc (Vladykov and Follett, 1967; Hubbs and Potter, 1971; Potter, 1980), trunk myomeres (Vladykov and Kott, 1979), and pigmentation (Potter and Osborne, 1975). All criteria of determinations were recently reviewed (Gardiner, 2003; Taverny and Élie, 2010).

As ammocoetes, lampreys have no mouth disc, no eyes and no developed fins. However, when over 50 mm long, *Petromyzon* ammocoetes can be distinguished from those of *Lampetra* by the number of trunk myomeres (Potter and Osborne, 1975) and different pigmentation patterns (Vladykov and Follett, 1967).

For the purposes of conservation management and vital habitats requirements (nursery areas), it seemed useful to differentiate without doubt young lamprey larvae, especially those under 60 mm in length. For this purpose, genetic tools are attractive.

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The most applied molecular markers for animal species identification, including “fish”, were based on the mitochondrial DNA sequences of cytochrome b (*cyt b*) and 16S rRNA (16S) genes (Kochzius, 2009; Teletchea, 2009). The European FishTrace consortium (www.fishtrace.org) used preferentially *cyt b* and the AnchovyID project generated partial 16S, *cyt b* and cytochrome oxidase I (*COI*) sequences for specimen identification (<https://anchovyid.jrc.ec.europa.eu/>). Later, an international consortium, “Barcoding of life”, chose *COI* gene for animal species identification (Hanner, 2009). However, Kochzius *et al.* (2010) concluded that *cyt b* and *COI* are equally well-suited for the sequence-based species identification of fish.

Our aim was to set up a fast and simple method to differentiate *P. marinus* from *L. fluviatilis* and *L. planeri* by using a Restriction Fragment Long Polymorphism (RFLP) based on *cyt b* sequence.

METHODS

Cyt b genes of *Petromyzon marinus* and *Lampetra* species were searched in databases (NCBI: www.ncbi.nlm.nih.gov). After sequence analysis, a single pair of primers was chosen in order to amplify a fragment of *Cyt b* in lampreys.

Lampreys were collected from various areas of the Gironde basin. Tissue samples were taken from nine *Lampetra planeri* subadult stages, 18 *L. planeri* adult stages, 17 *L. fluviatilis* adult stages and four *P. marinus* adult stages, in order to have no doubt regarding species identification for these stages. A piece of the upper region of the caudal fin was clipped from each individual and fixed in 90% ethanol.

DNA was extracted from 4 to 45 mg tissue using the QIAamp DNA mini kit (Qiagen, California, USA). Extraction efficiency was controlled by electrophoresis on a 1% agarose gel, running through 1X TBE buffer (Tris 89mM, EDTA 2mM, Borate 89 mM, Eurobio), stained by ethidium bromide (2 µg/ml) and read under an UV transilluminator (Vilber Lourmat), under 312 nm. A negative extraction control, using milliQ water instead of tissue, was added between every ten extractions.

Amplification of a 560 base pair fragment of *cyt b* was obtained using a polymerase chain reaction (PCR) with the following primers: *cyt b*-f (5'-CCTTCTCCT-GCTAATATCTC-3') and *cyt b*-r (5'-GGGTTACTAGATC-CTGTTTG-3') located in positions 76 to 95 and 635 to 616, respectively. 25 µl of PCR reaction contained: 10X buffer with 1.5 mM MgCl₂, 0.6 U of Taq DNA polymerase (Qbiogen, MP Biomedicals), 1.25 mM dNTP and 5 µM of each primer. Reactions were run in a Biorad thermocycler (Icycler) for 35 cycles consisting of denaturation at 94°C for 30 s, primer annealing at 50°C for 1 min, and extension at 72°C for 1 min. The 35 cycles were preceded by an initial

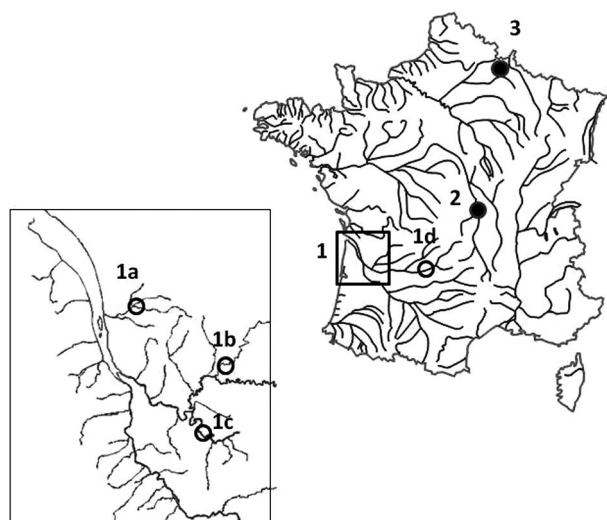


Figure 1. - Study stream locations where lamprey were collected. 1: Gironde basin; 1a: Livenne River; 1b: Dronne River; 1c: Dordogne River; 1d: Cere River; 2: Loire basin; 3: Meuse basin.

denaturation for 5 min at 94°C and followed by a final 5 min extension at 72°C. Negative controls, using milliQ water instead of template DNA, were included in each series of reactions to screen for possible contamination. PCR products were verified by 1.5% agarose gel electrophoresis. For some samples, PCR products were sequenced (Cogenics, France). The resulting sequences were analysed using NCBI/BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The PCR-RFLP method was carried out in order to differentiate *P. marinus* from *L. (fluviatilis-planeri)*. We started by an “in silico” restriction analysis to the 560 bp PCR fragment using bioinformatics tool (<http://molbiol-tools.ca>) and retrieve some RE that could be used. Then, the 560 bp fragment was digested by *Sau3A*, *EcoRI*, *TaqI*, *HindIII*, *RsaI*, *MspI*, *HinfI* (Promega) and the resulting fragments separated on a 2% agarose gel electrophoresis. Finally, RE *HinfI*, *RsaI* and *Sau3A* were chosen preferentially.

After this PCR-RFLP differentiation on adult or subadult lampreys, we used the same method to analyse 121 ammocoete larvae, collected from the Gironde basin (Livenne, Dronne, Cere and Dordogne rivers), the Loire River and the Rhin basin (Rule and Boulancourt rivers) (Fig. 1).

RESULTS

We found that PCR using the selected *cyt b*-f and *cyt b*-r primers produced a single band of 560 bp for the 20 samples analysed. The *cyt b* partial sequence analysis of seven *L. fluviatilis*, nine *L. planeri* and four *P. marinus* (adults and sub-adults) confirmed the species identification by phenotypic observations. Indeed, as demonstrated by other authors

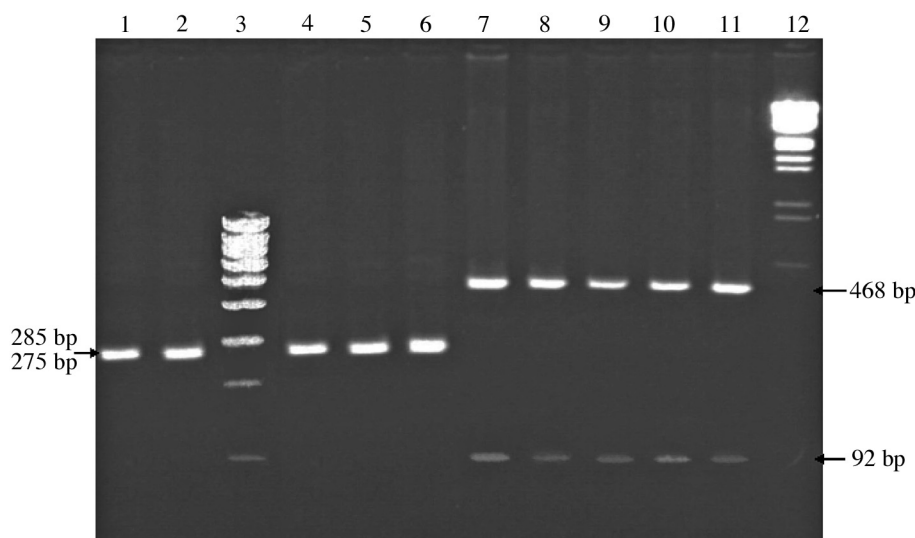


Figure 2. - PCR-RFLP results of the 560 bp *Cytb* fragment using *HinfI* restriction enzyme. Pistes 1-2: *L. fluviatilis*; 3: Ladder 100 bp; 4 to 6: *L. planeri*; 7 to 11: *P. marinus*; 12: Ladder Bacteriophage lambda digested by *EcoRI*-*HindIII*.

Table I. - Size of *L. (fluviatilis-planeri)* and *P. marinus* DNA fragments generated after RE (*Sau3A*, *EcoRI*, *RsaI*, *HinfI*) digestion of the 560 bp PCR fragment generated using the *cytb-f* and *cytb-r* primers.

Lamprey species	Restriction enzymes and size of fragments generated (bp)			
	<i>Sau3A</i>	<i>RsaI</i>	<i>HinfI</i>	<i>EcoRI</i>
<i>L. fluviatilis-planeri</i>	60	63	285	560
	180	164	275	
	320	333		
<i>P. marinus</i>	548	560	468	560
	12		92	

(Espanhol *et al.*, 2007; Blank *et al.*, 2008), discrimination between species pair *L. fluviatilis* and *L. planeri* is not possible using *cyt b* sequences. However, the *cyt b* of *P. marinus* presented sufficient differences to make species differentiation possible.

An example of profiles generated by PCR-RFLP is presented on figure 2. DNA fragments sizes obtained using the chosen RE are summarised in table I.

In fact, *RsaI* and *Sau3A* generated a one-band profile from *P. marinus* samples and a three-band profile from *L. (fluviatilis-planeri)* samples. Restriction using *HinfI* generated two bands from *P. marinus* samples and two bands (two of the bands migrated much closer together) from *L. (fluviatilis-planeri)* samples. By using two enzymes to analyse the same sample, the reliability of the method can be increased, but occasionally, for a rapid screening, one enzyme may be used (*HinfI*).

Out of the 121 ammocoete larvae collected from Gironde basin, Loire River, and Rhin basin, 73 were genetically determined as *L. (fluviatilis-planeri)* and 48 as *P. marinus* (Tab. II), using RE *HinfI*, *RsaI* and *Sau3A*.

Table II. - Phenotypic and genotypic (PCR-RFLP) identification of *L. (fluviatilis-planeri)* and *P. marinus* ammocoetes isolated from various French rivers, using RE *Sau3A*, *RsaI* and *HinfI*.

Basins	Rivers	N	Median length (mm)	Phenotypic identification		Genotypic identification		RE
				<i>P. marinus</i>	<i>L. fluviatilis-planeri</i>	<i>P. marinus</i>	<i>L. fluviatilis-planeri</i>	
Gironde	Livonne	19	32	2	17	2	17	<i>RsaI</i> , <i>HinfI</i>
	Dronne	11	60	6	5	7	4	<i>RsaI</i> , <i>HinfI</i>
	Dordogne	19	33	6	13	18	1	<i>RsaI</i> , <i>HinfI</i>
	Cere	23	24	19	4	19	4	<i>RsaI</i> , <i>HinfI</i>
Loire	Loire	2	103	2	0	2	0	<i>Sau3A</i> , <i>RsaI</i> , <i>HinfI</i>
Meuse	Rule	28	107	1	27	1	27	<i>RsaI</i> , <i>HinfI</i>
	Boulacourt	19	94	0	19	0	19	<i>Sau3A</i> , <i>RsaI</i> , <i>HinfI</i>
Total		121	62	36	85	49	72	

DISCUSSION

Ours results demonstrated that *P. marinus* *cyt b* sequence was easily differentiated from those of *L. (fluviatilis-planeri)*. However, the two *Lampetra* species cannot be separated using *cyt b* sequences. We regarded the possibility to use the *COI* gene utilised in the “Barcoding of life” program. An “in silico” analysis of *COI* sequences demonstrated the impossibility to differentiate *L. fluviatilis* and *L. planeri* (pers. data; April *et al.*, 2011). Moreover, we generated various sequences of the most variable mitochondrial region (D-loop) and demonstrated the failure to discriminate the two species of *Lampetra* (data not shown). Okada *et al.* (2010) obtained the same results for the lamprey. The genetic study carried out by Espanhol *et al.* (2007) supported our results about *cyt b*. More recently, studying the brook lampreys from Pacific coast of North America, Boguski (2009) retrieved that, on average, *cyt b* evolves 1.43 fold faster than *COI* and concluded that the two genes may be used in species differentiation.

In order to carry out a rapid differentiation between *P. marinus* and *L. (fluviatilis-planeri)*, a 560 bp fragment was chosen in the most variable region of *cyt b* sequence, and a RFLP was applied using selected RE. This genetic differentiation method can be carried out in less than 48 h. The classic phenotypic keys first used to differentiate the larvae were in agreement with the genotypic test in 81% of cases (Tab. II), but only when using larvae over 60 mm length. In contrast, using these phenotypic tests provided numerous differentiation errors with larvae under 60 mm. The majority of these errors (93%) concerned *Lampetra* sp. instead of *P. marinus*: 45% larvae living in Dronne River were 22 to 60 mm length and 95% larvae from Dordogne River were 25 to 40 mm. But there were no error made with larvae from Livenne River where 74% of them were under 60 mm. Factors like food could influence the intensity of larvae pigmentation and mislead the phenotypic species determination.

This genotypic test is useful to assess characteristics like external features used during early life stages (e.g. eggs, larvae) of sympatric species, including lampreys (Mandrak *et al.*, 2004; Neave *et al.*, 2007). It can also help in improving ecological surveys such as studies of population structure in larval lamprey species (Yamazaki *et al.*, 2003) and their habitat use (Yamazaki, 2007).

In the field, larvae must be anaesthetised before clipping a piece of the upper region of the caudal fin (3 mm²), and the samples must be preserved in ethyl alcohol prior to be transported to the laboratory.

This work allowed research about population repartition of lamprey larvae on the scale of a river or a catchment basin. We could consider implementing protective zones corresponding to larva settlements on different spatial scales. This

implementation could allow refining the actual management plans, essentially based on spawning area protection.

It could be interesting to examine the lowest extracted DNA solution concentrations needed for efficient PCR amplifications in order to spare the young-of-the-year larvae a lethal or disabling biopsy.

Such a convenient and robust method of species identification for *P. marinus* and *L. (fluviatilis-planeri)* larvae is certainly an improvement compared to the traditional phenotypic key method.

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